

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



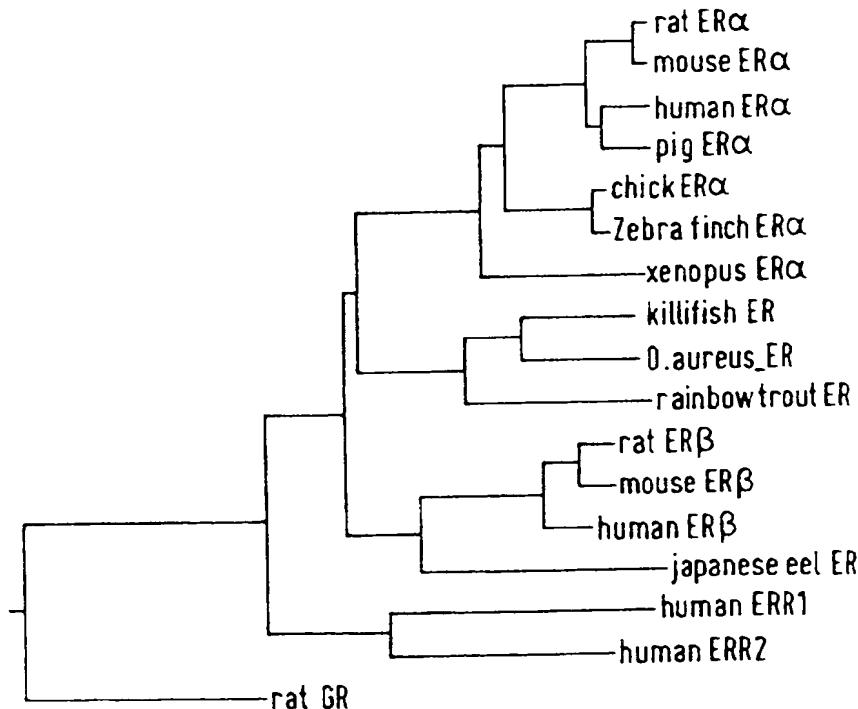
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/705		A2	(11) International Publication Number: WO 97/09348
(21) International Application Number:		PCT/EP96/03933	(43) International Publication Date: 13 March 1997 (13.03.97)
(22) International Filing Date:		9 September 1996 (09.09.96)	(81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(30) Priority Data:			Published <i>Without international search report and to be republished upon receipt of that report.</i>
9518272.1 9605550.4 9607532.0 9609576.5		8 September 1995 (08.09.95) 15 March 1996 (15.03.96) 11 April 1996 (11.04.96) 8 May 1996 (08.05.96)	GB GB GB GB
(71) Applicant (for all designated States except US): KARO BIO AB [SE/SE]; Novum, S-141 57 Huddinge (SE).			
(72) Inventors; and			
(75) Inventors/Applicants (for US only): KUIPER, George, G., J., M. [SE/SE]; Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Novum, S-141 86 Huddinge (SE). ENMARK, Eva [SE/SE]; Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Novum, S-141 86 Huddinge (SE). GUSTAFSSON, Jan-Ake [SE/SE]; Center for Biotechnology and Department of Medical Nutrition, Korolinska Institute, Novum, S-141 86 Huddinge (SE).			
(74) Agent: DEAN, John, Paul; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2JT (GB).			

(54) Title: ORPHAN RECEPTOR

(57) Abstract

This invention relates to a novel estrogen receptor-related nuclear receptor, hereinafter termed "ER β " having the amino acid sequence of Figs. 1, 13A or 14A or substantially the same amino acid sequence as the amino acid sequence shown in Figs. 1, 13A or 13B or an amino acid sequence functionally similar to that sequence. The invention also relates to DNA sequences encoding the receptor. The receptor may be useful in isolating molecules for the treatment of disorders such as prostate cancer, benign prostatic hyperplasia, osteoporosis or cardiovascular disorders and in the testing of substances for estrogenic and other hormonal effects.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

ORPHAN RECEPTOR

This invention relates to cellular nuclear receptors and their uses..

A large family of nuclear receptors which confer cells with responsiveness to molecules such as retinoid acid, vitamin D, steroid hormones and thyroid hormones has been identified. Extensive studies have shown that the members of this superfamily of nuclear receptors activate and/or repress gene transcription through direct binding to discrete *cis*-acting elements termed "hormone response elements" (HRE). It has been shown that these HRE's comprise repeats of consensus palindromic hexanucleotide DNA motifs. The specificity of the HRE's is determined by the orientation of, and spacing between, halfsites (i.e. half a palindromic sequence)(Umenesono K., *et al*, 1991 *Cell* **65**, 1255-1266).

Specific DNA binding is mediated by a strongly-conserved DNA binding domain, containing two zinc fingers, which is conserved among all thus discovered nuclear receptors. Three amino acids at the C-terminal base of the first zinc finger (known as the "P-box") are important for the recognition of the half site nucleotide sequence. Members of the nuclear receptor superfamily have been classified into different groups on the basis of the amino acid sequence within the P box.

All members of the nuclear receptor superfamily also contain a hypervariable N-terminal domain and a ligand-binding domain containing some "patches" of conserved sequence. One of these is called the "Ti-domain".

Molecules which are thought to be nuclear receptors, as they are structurally related to characterised receptors, but for which no ligand has been found, are termed "orphan receptors". Many such orphan receptors have been identified (see for example Evans R.M. (1988) *Science* **240**, 889-895 and O'Malley, B. (1990) *Mol. Endocrinol.* **4** 363-369)

We have now unexpectedly identified, initially in rat a new orphan receptor, which is related to the known estrogen receptor ER α , and which we have designated "ER β " (specifically "rER β " in rat). In this specification "Er β " will be used to refer to the receptors hER β or rER β or related receptors. The nucleotide and amino acid sequences of rER β have now been determined and are shown in Fig. 1. We have also identified a human Er β - "hER β ", the amino acid DNA and sequences of which are shown in Fig. 13A and 13B respectively.

According to one aspect of the invention there is provided a novel estrogen receptor-related nuclear receptor, hereinafter termed "ER β " having the amino acid sequence of Figs. 1, Fig. 13A or 16A or substantially the same amino acid sequence as the amino acid sequence shown in Figs. 1, 13A or 16A or an amino acid sequence functionally similar to those sequence. The isolated receptor may be particularly useful in the search for molecules for use in treatment of diseases or conditions such as cardiovascular diseases, central nervous system diseases or conditions or osteoporosis, prostate cancer or benign prostatic hyperplasia.

The receptor of the invention may also be used in the testing of environmental chemicals for estrogenic activity. There has been increasing concern over the effect of various

chemicals released into the environment on the reproduction of humans and animals.

Threats to the reproductive capabilities of birds, fish, reptiles, and some mammals have become evident and similar effects in humans have been proposed. Substantial evidence is now emerging which shows that exposure to certain chemicals during critical periods of foetal life may distort the development of the reproductive organs and the immune and nervous systems. On the basis of possible parallels between actual wildlife effects, seen for example in birds and seals living in highly polluted areas, and proposed effects in humans, in combination with documented human reproductive effects caused by prenatal exposure to the pharmaceutical estrogen, diethyl stilbestrol (DES), "estrogenic" chemicals have been proposed to threaten the reproductive capability of both animals and humans.

Among the chemicals known or suspected to act as estrogen mimics on the human body, or in other ways disturb the human endocrine system, there are several which have already been identified as environmental hazards. Among the chemicals that have been mentioned as potential causes of disruption of reproductive function in animals and humans are chlorinated organic compounds such as dieldrin, endosulfans, chlordanes, endrins, aldrin, DDT and some PCBs, plastics such as Bisphenol A, phthalates and nonylphenol, and aromatic hydrocarbons. Some of the proposed effects on humans have been suggested to be due to an increasing exposure to environmental estrogens - in fact, exposure to chemical compounds to which higher organisms during the foetal period react in a way that is similar to when they are exposed to high dosages of estrogens. The effects are manifested by for example perturbations of the sex characteristics and impaired reproductive potential. In humans, elevated risks of breast cancer and other hormone-related disease has also been discussed as possible effects. In addition, to the documented "estrogenic" effects, it has recently been demonstrated that environmental

pollutants may also act on hormonal pathways other than the estrogenic pathway - it has been shown that p,p' - DDE the main metabolite of DDT (also in humans) is a fairly anti-androgenic agent (Kelce W.R. *et al* Nature 1995 375:581-585). Epidemiological studies on these issues are, however, presently difficult to interpret. Nevertheless, there is a growing opinion against these potentially hormone disrupting chemicals, and very palpable public and environmental demand for the governmental agencies and industry to act. In view of the similarities between the receptor of the present invention, Er β and the classical estrogen receptor, Er β may be used in the testing of chemicals for estrogenic effect.

An amino acid sequence functionally-similar to the sequence shown in Fig. 1, 13A or 14A may be from a different mammalian species.

An amino acid sequence which is more than about 89% identical with the sequence shown in Fig. 1, 13A or 14A is substantially the same amino acid sequence for the purposes of the present application. Preferably, the amino acid sequences is more than about 95% identical with the sequence shown in Fig. 1, 13A or 14A.

According to another aspect of the invention there is provided a DNA sequence encoding a nuclear receptor according to the first aspect of the invention. Preferably, the DNA sequence is that given in Fig. 1, 13A or 14A or is a DNA sequence encoding a protein or polypeptide having the functionality of ER β .

ER β is unique in that it is extremely homologous to the rat estrogen receptor, in particular in its DNA binding domain. It appears that ER β has a very limited tissue distribution. In

female rats, it appears to be present only in the ovaries, and in male rats in the prostate and testes. As these tissues are classic targets for estrogen action, it can be deduced that ER β may mediate some of the effects of estrogen.

The different ligand specificity of ER α and ER β may be exploited to design pharmaceutical agents which are selective for either receptor. In particular, the differences in ligand specificity may be used to develop drugs that specifically target cardiovascular disease in postmenopausal women or osteoporosis.

The nuclear receptor of the invention, ER β , a method of producing it, and tests on its functionality will now be described, by way of example only, with reference to the accompanying drawings, Figs. 1 to 15 in which:

Fig. 1 shows the amino acid sequence of ER β and the nucleotide sequence of the gene encoding it:

Fig. 2A is a phylogenetic tree showing the evolution of ER β and other receptors;

Fig. 2B shows the homology between the different domains in ER β and certain other receptors;

Fig. 2C is an alignment of the amino acid sequence in the ligand binding domains of rER β , rER α , mER α and hER α :

Fig. 2D is an alignment of the amino acid sequence in the DNA binding domains of rER β , rER α , mER α and hER α :

Fig. 3A is a film autoradiograph of prostate gland showing strong expression of a clone of the receptor of the invention, clone 29;

Fig. 3B is a darkfield image showing prominent signal for clone 29 in epithelium (e) of prostatic alveoli. The stroma(s) exhibit(s) weaker signal;

Fig. 3C is a bipolarization image of cresyl violet counterstained section showing silver grains over epithelium (e), whereas the stroma(s) contain(s) less grains;

The bar represents 0.7 mm for Fig. 3A. 200 μm for Fig. 3B and 30 μm for Fig. 3C;

Fig. 4A shows a film autoradiograph of ovary showing strong expression of clone 29 in follicles at different developmental stages (some are indicated by arrows). The interstitial tissue (arrowheads) shows low signal;

Fig. 4B shows a darkfield image showing high expression of clone 29 in granular cells of primary (1), secondary (2), tertiary (3) and mature (4) follicles. Low signal is present in interstitial tissue (it);

Fig. 4C is a bipolarization image of ovary showing strong signal in granular cells (gc), whereas the oocyte (oc) and the cainterna (ti) are devoid of clear signal;

The bar represents 0.9 mm for Fig. 4A. 140 μm for Fig. 4B and 50 μm for Fig. 4C;

Fig. 5A illustrates the results of saturation ligand binding analysis of cloned ER β ;

Fig. 5B illustrates the specificity of ligand binding by cloned ER β ;

Fig. 5C illustrates E2 binding by ER β ;

Fig. 6 illustrates the activation of transcription by cloned ER β ;

Fig. 7 and 7A illustrates stimulation by various ligands by cloned ER β ;

Fig. 8 illustrates the results of RT-PCR experiments on the expression of rat estrogen receptors:

Fig. 9 illustrates the results of RT-PCR experiments on the expression of human Er β (hER β);

Fig. 10A is a Hill plot comparing binding of ^{125}I -E2 by hER α and rER β :

Fig. 10B is a Scatchard plot comparing binding of ^{125}I -E2 by hER α and rER β .

Fig. 11A illustrates the relative binding affinity of hER α and rER β for various ligands:

Fig. 11B is a detail of Fig. 12A;

Fig. 12 is an alignment of various estrogen receptors:

Fig. 13A shows the amino acid sequence of human ER β :

Fig. 13B shows the DNA sequence of human Er β :

Fig. 14A shows the amino acid sequence of mER β .

Fig. 14B shows the DNA sequence of mouse Er β : a

Fig. 15 illustrates ligand binding affinities for various phytoestrogens by ER's of the invention.

A. CLONING OF RAT ER β

i. PCR-amplification and complementary DNA cloning.

A set of degenerate primers (DBD 1,2,3 and WAK/FAK) were designed previously according to the most highly conserved sequences of the DNA-binding domain (P-box) and ligand binding domain (Ti-stretch) of members of the nuclear receptor family (Enmark, E., Kainu, T., Peito-Huikko, M., & Gustafsson, J.-Å (1994)

Biochem. Biophys. Res. Commun. **204**, 49-56). Single strand complementary DNA reverse transcribed from rat prostate total RNA was employed with the primers in PCR reactions as described in Enmark, E., Kainu, T., Pelto-Huikko, M., & Gustafsson, J.-Å (1994) *Biochem. Biophys. Res. Commun.* **204**, 49-56. The

SUBSTITUTE SHEET (RULE 26)

amplification products were separated on a 2% low melting agarose gel and DNA products between 400 and 700 bp were isolated from the gel and ligated to TA cloning vector (Invitrogen). As alternatives, we also used the RP-I/RP-2 and DBD66-100/DBD210-238 primer sets in the DNA-binding domain of nuclear receptors exactly as described by Hirose T., Fijimoto, W., Yamaai, T., Kim, K.H., Matsuura, H., & Jetten, A.M (1994) *Mol. Endocrinol.* **8**, 1667-1677 and Chang, C., Lopes Da Silva, S., Ideta, R., Lee, Y., Yeh, S., & Burbach, J.P.H (1994) *Proc. Natl. Acad. Sci.* **91**, 6040-6044 respectively. Clone number 29 (obtained with the DBD-WAK/FAK set) with a length of 462 bp showed high homology (65%) with the rat estrogen receptor cDNA (65%), which was previously cloned from rat uterus (Koike, S., Sakai, M., & Muramatsu, M. (1987) *Nucleic Acids Res* **15**, 2499-2513). The amino acid residues predicted by clone 29 DNA sequences suggested that this DNA fragment encoded part of the DNA-binding domain, hinge region and the beginning of the ligand binding domain of a novel member of the nuclear receptor family. Two PCR primers (Figure 1) were used to generate a probe of 204 bp consisting of the hinge region of the novel receptor, which was used to screen a rat prostate cDNA library (Clontech gt10) under stringent conditions resulting in four strongly positive clones with a size of 0.9 kb, 1.8kb, 2.5kb and 5-6kb respectively. The clone of 2.5kb was sequenced and Figure 1 shows the nucleotide sequence determined in the core facility (CyberGene AB) by cycle sequencing using fluorescent terminators (Applied Biosystems) on both strands, with a series of internal primers and deduced amino acid sequence of clone 29. Two in frame ATG codons are located at nucleotide 424 and nucleotide 448, preceding by an in-frame stop codon at nucleotide 319, which suggests that they

are possible start codons. The open reading frame encodes a protein of 485 amino acid residues (counted from the first methionine) with a calculated molecular weight of 54.2 kDa. Analysis of the proteins synthesized by *in-vitro* translation from the clone 29 cRNA in rabbit reticulocyte lysate revealed a doublet protein band migrating at approximately 57 kDa on SDS-PAGE gels (data not shown), confirming the open reading frame. The doublet protein band is probably caused by the use of both ATG codons for initiation of protein synthesis. The amino acid sequence of clone 29 protein shows the characteristic zinc module DNA-binding domain, hinge region and a putative ligand binding domain, which are the characteristic features of members of the nuclear receptor family (Tsai, M-J., & O'Malley, B.W (1994) *Ann. Rev. Biochem.* **63**, 451-486; Härd, T., & Gustafsson, J.-Å (1993) *Acc. Chem. Res.* **26**, 644-650; Laudet, V., Hänni, C., Coli, J., Catzeffis, F., & Stehelin, D (1992) *EMBL J.* **11**, 1003- 1012).

Protein sequence comparison with several representative members of the nuclear receptor family (Figure 2) showed the clone 29 protein is most related to the rat estrogen receptor (ER α). cloned from uterus (Koike, S.. Sakai, M.. & Muramatsu. M. (1987) *Nucleic. Acids Res.* **15**, 2499-2513), with 95% identity in the DNA-binding domain (amino acid residues 103-167) (Griffiths, K., Davies, P., Eaton, C.I., Harper, M.E., Turkes, A., & Peeling, W.B. (1991) in *Endocrine Dependent Tumours*, eds. Voigt, K-D. & Knabbe, C. (Raven Press), pp. 83-125).

A number of functional characteristics have been identified within the DNA-binding domain of nuclear receptors (Härd, T., & Gustafsson, J.-Å. (1993) *Acc. Chem. Res.* **26**, 644-650 and Zilliacus, J.. Carlstedt-Duke, J., Gustafsson, J.-Å.. & Wright, A.P.H. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 4175-4179). The

so-called P-box specifies nucleotide sequence recognition of the core half-site within the response element, while the D- box mediates dimerization between receptor monomers. The clone 29 protein P-box and D-box sequences of EGCKA and P.ATNQ, respectively, are identical to the corresponding boxes in ER α (Härd, T., & Gustafsson, J.-Å. (1993) *Acc. Chem. Res.* **26**, 644-650 and Koike, S., Sakai, M., & Muramatsu, M. (1987) *Nucleic Acids Res.* **15**, 2499-2513), thus predicting that clone 29 protein binds to ERE sequences.

The putative ligand binding domain (LBD) of clone 29 protein (amino acid residues 259-457) shows closest homology to the LBD of the rat ER α (Figure 2), while the homology with the human ERR1 and ERR2 proteins (Giguere, V., Yang, N., Segui, P., & Evans R.M. (1988) *Nature* **331**, 91-94) is considerably less. With the human, mouse and xenopus estrogen receptors the homology in the LBD is also around 55%, while the homology with the LBD of other steroid receptors is not significant (Figure 2). Cysteine residue 530 in human ER α has been identified as the covalent attachment site of an estrogenic affinity label (Harlow, K.W., Smith D.N., Katzenellenbogen, J.A., Greene, G.L., & Katzenellenbogen, B.S. (1989) *J. Biol. Chem.* **264**, 17476- 17485). Interestingly, clone 29 protein (Cys-436) as well as the mouse, rat and xenopus ER α s have a cysteine residue at the corresponding position. Also, two other amino acid residues described to be close to or part of the ligand-binding pocket of the human ER α -LBD (Asp 426 and Gly 521) are conserved in the LBD of clone 29 protein (Asp 333 and Gly 427) and in the LBD of ER α s from various species (20,21). The ligand-dependent transactivation function TAF-2 identified in ER α (Danielian, P.S., White, R., Lees, J.A., & Parker,

M.G. (1992) *EMBO J.* **11**, 1025-1033), which is believed to be involved in contacting other transcription factors and thereby influencing activation of transcription of target genes. is almost completely conserved in clone 29 protein (amino acid residues 441-457). Steroid hormone receptors are phosphoproteins (Kuiper, G., & Brinkmann, A.O. (1994) *Mol. Cell. Endocrinol.* **100**, 103-107), and several phosphorylation sites identified in the N-terminal domain and LBD of ER α (Arnold, S.F., Obourn, J.D., Jaffe, H., & Notides, A.C. (1995) *Mol. Endocrinol.* **9**, 24-33 and Le Goff, P., Montano, M.M., Schodin, D.J., & Katzenellenbogen, B.S (1994) *J. Biol. Chem.* **269**, 4458-4466) are conserved in clone 29 protein (Ser 30 and 42, Tyr 443). Clone 29 protein consists of 485 amino acid residues while ER α s from human, mouse and rat consist of 590-600 amino acid residues. The main difference is a much shorter N-terminal domain in clone 29 protein i.e 103 amino acid residues as compared to 185-190 amino acid residues in the other receptor proteins. Also the non-conserved so-called F-domain at the C-terminal end of ER α s is 15 amino acid residues shorter in clone 29 protein. The cDNA insert of a positive clone of 2.6 kb was subcloned into the EcoRI site of pBluescript (trademark) (Stratagene). The complete DNA sequence of clone 29 was determined (CyberGene AB) by cycle sequencing using fluorescent terminators (Applied Biosystems) on both strands, with a series of internal primers.

Figs 2C and 2D respectively compare the ligand and DNA binding domain of Er β compared to rat, mouse and human Er α 's.

2. Saturation ligand binding analysis and ligand competition studies:

Clone 29 cDNA was subcloned in pBluescript downstream of the T7 promoter to give p29-T7. Clone 29 protein was synthesized *in vitro* using the TnT-coupled reticulocyte lysate system (Promega). Translation reaction mixtures were diluted five times with TEDGMO buffer (40 mM Tris/HCl, pH 7.4, 1mM EDTA, 10% (v/v) glycerol, 10 mM Na₂MoO₄, 10 mM DTT) and 0.1 ml aliquots were incubated for 16 h at 8°C with 0.3- 6.2 nM [2,4,6,7-³H]-17β-estradiol (NEN-Dupont; specific radioactivity 85 Ci/mmol) in the presence or absence of a 200-fold excess of unlabelled E2.

Fig. 5A illustrates the results of a saturation ligand analysis of clone 29 protein. Reticulocyte lysate containing clone 29 protein was incubated with 6 concentrations of [³H]E2 between 0.3 and 6.0 nM. Parallel tubes contained an additional 200 fold of non-radioactive E2. Bound and free ligand were separated with a dextran-coated charcoal assay. The Kd (0.6 nM) was calculated from the slope of the line in the Scatchard plot shown ($r = 0.93$), and the number of binding sites was extrapolated from the intercept on the abscissa (Bmax = 1400 fmol/ml undiluted translation mixture).

For ligand competition studies diluted reticulocyte lysate was incubated with 5 nM [2,4,6,7-³H]-17β-estradiol in the presence of either 0, 5, 50, 500 or 5,000 nM of non-radioactive E2, estrone, estriol, testosterone, progesterone, corticosterone, 5α-androstan-3β,17β-diol, 5α-androstan-3α,17β-diol and diethylstilbestrol (DCES) for 16 h at 8°C. Bound and unbound steroids were separated with a dextran-coated

charcoal assay (Ekman, P., Barrack, E.R., Greene, G.L., Jensen, E.V., & Walsh, P.C (1983) *J. Clin. Endocrinol Metab.* **57**, 166-176).

Fig. 5B illustrates the specificity of ligand binding by clone 29 protein. Reticulocyte lysate containing clone 29 protein was equilibrated for 16 h with 5 nM [³H]E2 and the indicated fold excess of competitors. Data represent [³H]E2 bound in the presence of unlabelled E2, testosterone (T), progesterone (prog), corticosterone (cortico), estrone (E1), diethylstilbestrol (DES), 5 α -androstane-3 α , 17 β -diol (3 α -AD), 5 α -androstane- 3 β ,17 β -diol (3 β -AD) and estriol (E3). [³H]E2 binding in the absence of competitor was set at 100%.

3. *In-situ hybridisation:*

In-situ hybridisation was carried out as previously described (Dagerlind Å., Friberg, K., Bean, A.J., & Hökfelt, T (1992) *Histochemistry* **98**, 39-49). Briefly, two oligonucleotide probes directed against nucleotides 994-1041 and 1981-2031 were each labelled at the 3'-end with ³²P-dATP using terminal deoxynucleotidyltransferase (Amersham, UK). Adult male and female Sprague-Dawley rats (age 2 to 3 months n=10) were used for this study. The rats were decapitated and the tissues were rapidly excised and frozen on dry ice. The tissues were sectioned in a Microm HM500 cryostat at 14 μ m and thawed onto Probe-On glass slides (Fisher Scientific, PA, USA). The slides were stored at -20°C until used. The slides were incubated in humidified boxes at 42°C for 18 h with 1x10⁶ cpm of the probe in a hybridization solution containing 50% formamide, 4 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1 x

Denhardt (0.02 % BSA, 0.02 % Ficoll, 0.02 % PVP), 1 % sarkosyl, 0.02 M sodium phosphate (pH 7.), 10% dextran sulphate, 500 µg/ml salmon sperm DNA and 200 mM DTT. Slides were subsequently rinsed in 1 x SSC at 55°C for 60 min with four changes of SSC and finally in 1 x SSC starting at 55°C and slowly cooled to room temperature, transferred through distilled water and briefly dehydrated in 50% and 95% ethanol for 30 sec each, air-dried, and covered with Amersham β-man autoradiography film for 15 to 30 days. Alternatively the slides were dipped in Kodak NTB2 nuclear track emulsion (diluted 1:1 with distilled water) and exposed for 30 to 60 days at 4°C. Finally, the sections were stained with cresyl violet.

Clear expression of clone 29 was observed in the reproductive tract of both male and female rats, while in all other rat tissues the expression was very low or below the level of detection with *in-situ* hybridisation (not shown). In male reproductive organs high expression was seen in the prostate gland (Figure 3), while very low expression was observed in testis, epididymis and vesicula seminalis (not shown). In dipped sections, expression was clearly visible in prostate epithelial cells (secreting alveoli) while the expression in smooth muscle cells and fibroblasts in the stroma was low (Figure 3). In female reproductive organs expression was seen in the ovary (Figure 4), while uterus and vagina were negative (not shown). In dipped sections high expression was seen in the granulosa cell layer of primary, secondary and mature follicles (Figure 4), whereas primordial follicles, oocytes and corpora lutea appeared completely negative. Low expression was seen in the interstitial cells of the ovary. Both anti-sense oligonucleotide probes used

produced similar results. Addition of a 100 fold excess of the respective unlabelled oligonucleotide probes during the hybridisation reactions abolished all signals.

4. **Transactivation analysis in CHO-cells:**

The expression vector pCMV29 was constructed by inserting the 2.6 kb clone 29 fragment in the EcoRI site of the expression vector pCMV5 (Andersson, S., Davis, D.L., Dahlbäck, H., Jörnvall, H., & Russell, D.W. (1989) *J. Biol. Chem.* **264**, 8222-8229). The pERE-ALP reporter construct contains a secreted form of the placental alkaline phosphatase gene (Berger, J., Hauber, J., Hauber, R., Geiger, R., & Cullen, B.R. (1988) *Gene* **66**, 1-10) and the MMTV-LTR in which the glucocorticoid response elements were replaced by the vitellogenin promoter estrogen response element (ERE).

CHO-K1 cells were seeded in 12-well plates at approximately 1.7×10^5 cells per well in phenol-red free Ham F12 medium with 5% FCS (dextran-coated charcoal treated) and 2 mM Lglutamine. After 24 h the cells were transfected with 250 ng pERE-ALP vector and 50 ng pCMV29 using lipofectamine (Gibco) according to the manufacturer's instructions. After five hours of incubation the cells were washed and refed with 0.5 ml phenol-red free Coon's F-12 medium containing 5% serum substitute (SRC 3000, Tissue Culture Services Ltd., Botoiph Claydon, Buckingham, UK) 2 mM Lglutamine and 50 µg/ml gentamicin plus hormones as indicated. After 48 h the medium was assayed for alkaline phosphatase (ALP) activity by a chemiluminescence assay. A 10 µl aliquot of the cell culture medium was mixed with 200 µl assay buffer (10 mM diethanolamine pH 10.0 1 mM MgCl₂ and 0.5 mM CSPD (Tropix Inc. Boston, USA)) and incubated for 20 min at 37°C

before measurement in a microplate luminometer (Luminoskan: Labsystems, Finland) with integral measurement for 1 second. The ALP activity of ERE-reporter alone was set at 1.

5. **Ligand binding characteristics and transactivation function of clone 29 protein:**

On the basis of the described high homology between clone 29 protein and rat ER α in the DBD and LBD it was hypothesized that clone 29 protein might encode a novel ER. Furthermore, biological effects of estrogens on rat prostate and ovary, which show high expression of clone 29 RNA, are well known (Griffiths, K., Davies, P., Eaton, C. I., Harper, M.E., Turkes, A., & Peeling W. B. (1991) in *Endocrine Dependent Tumours*, eds Voigt, K-D. & Knabbe, C. (Raven Press), pp 83-125; Richards, J.S (1994) *Endocrine Rev.* **15**, 725-745; and Habenicht, U-F., Tunn, U.W., Senge, Th., Schroder, R.H., Schweikert, H.U., Bartsch, G., & El Etreby, M.F. (1993) *J. Steroid Biochem. Molec. Biol.* **44**, 557-563). In order to analyze the steroid binding properties of clone 29 protein synthesized *in vitro*, the reticulocyte lysate was incubated at 8°C for 16 h with increasing concentrations (0.3-6.0 nM) of [3 H]E2 in the presence or absence of a 200 fold molar excess of unlabelled E2. Linear transformation of saturation data revealed a single population of binding sites for E2 with a K_d (dissociation constant) of 0.6 nM (Figure 5A and C). Steroid binding specificity was measured by incubating reticulocyte lysate with 5 nM [3 H]E2 in the presence of 0.5, 50, 500 and 5,000 nM unlabelled competitors. Competition curves generated are indicative of an estrogen receptor in that only estrogens competed efficiently with [3 H]E2 for binding

(Figure 5B). Fifty percent inhibition of specific binding occurred by 0.6 fold excess of unlabelled E2; diethylstilbestrol, estriol, estrone and 5 α -androstane-3 β ,17 β -diol were 5, 15, 50 and 150 times, respectively, less effective as competitors. Neither testosterone, progesterone, corticosterone nor 5 α - androstane-3 α ,17 β -diol were efficient competitors, even at the highest concentrations used (1000 fold excess). The dissociation constant and the steroid binding specificities measured are in good agreement with data previously reported for ERs in rat and human prostate, rat granulosa cells, rat antral follicles and whole rat ovarian tissue (Ekman, P., Barrack, E.R., Greene, G.L., Jensen, E.V., & Walsh, P.C (1983) *J. Clin. Endocrinol. Metab.* **57**, 166-176; van Beurden-Lamers, W.M.O., Brinkmann, A.O., Mulder, E., & van der Molen, H. (1974) *Biochem. J.* **140**, 495-502; Kudolo, G.B., Elder, M.G., & Myatt, L. (1984) *J. Endocrinol.* **102**, 83-91; and Kawashima, M., & Greenwald, G.S. (1993) *Biology of Reprod.* **48** 172-179).

When clone 29 protein was labelled with a saturating dose of [³H]E2 and analyzed on sucrose density gradients, a single peak of specifically bound radioactivity was observed. The sedimentation coefficient of this complex was about 7S, and it shifted to 4S in the presence of 0.4 M NaCl (not shown). To investigate the transcriptional regulatory properties of clone 29 protein, we performed co-transfection experiments in which CHO cells were transfected with a clone 29 protein expression vector and/or an estrogen-responsive reporter gene construct. Cells were incubated in the absence of E2 (clone 29) or in the presence of 100 nM E2 (Clone 29 + E2) or in the presence of 100 nM E2 and 12 μ M Tamoxifen (Clone 29 + E2/Tam). In the absence of exogenously added E2 clone 29 protein showed

considerable transcriptional activity which could be further increased by the addition of 100 nM E2 (Figure 6). Simultaneous addition of a 10 fold excess of the antiestrogen Tamoxifen partially suppressed the E2 stimulated activity (Figure 6). The constitutive transcriptional activity of clone 29 protein could be suppressed by the anti-estrogen ICI-1624384 (not shown). It has been shown previously that the wild-type mouse and human ERs are constitutive activators of transcription, and that the transcriptional activity can be stimulated further by the addition of E2 (Txukerman, M., Xiao-Kun Zhang., Hermann, T., Wills, K. N., Graupner, G., & Phal, M. (1990) *New Biologist* **2**, 613-620 and Lees, J.A., Fawell, S.E., & Parker, M.G. (1989) *Nucl. Acids Res.* **17**, 5477-5488). To obtain more insight into what concentrations of E2 effect clone 29 protein transcriptional activity, transient transfection experiments were carried out in the presence of increasing concentrations of E2. CHO-cells were transiently transfected with the ERE-reporter plasmid and the clone 29 protein expression plasmid. Cells were incubated with increasing concentrations of E2 (0.1 - 1000 nM), estrone (E1, 1000 nM), 5 α -androstane-3 β ,17 β -diol (3 β -AD, 1000 nM) or no ligand added. Alkaline phosphatase activity (ALP) was measured as described and the activity in the absence of ligand (control) was set at 1. The figure shows relative ALP-activities (=SD) from three independent experiments. Clone 29 protein began to respond at 0.1 nM E2 and maximal stimulation was observed between 1 nm and 10 nM E2 (Figure 7). The maximal stimulation factor was 2.6 = 0.5 fold (mean = SD, n = 9) as compared to incubation in the absence of E2. Apart from E2 also estrone and 5 α -androstane- 3 β ,17 β -dioi could stimulate transcriptional activity, albeit at higher concentrations (Figure 7). Dexamethasone, testosterone, progesterone,

$\Delta\alpha$ -androstane- β , α , β -diol, thyroid hormone and all-*trans*-retinoic acid could not stimulate transcriptional activity of clone 29 protein, even at the highest concentration (1000 nM) tested (not shown). The results of the co-transfection experiments are in agreement with the ligand binding and specificity data of clone 29 protein presented in Figure 5. In control experiments, wild-type human ER α also showed transcriptional activity in the absence of E2, which could be increased by the addition of E2 (not shown).

6. Detection of rat ER expression by RT-PCR

The tissue specificity of expression of rat ER β and ER α was determined using reverse transcriptase polymerase chain reaction (RT-PCR). The results of the experiment are shown in Fig. 8.

B. Isolation of human Er β

I. A human version of Er β (hER β) has also been cloned from human ovary. The tissue specificity of hER β expression in a variety of cells was also determined using the RT-PCR technique. The results are shown in Fig. 9. It will be noticed that there is a very high level of mRNA of hER β in human umbilical vein endothelial cells (HUVEC) but no detection of hER α in the same cells. In addition, it will be seen that in human osteosarcoma cell line (HOS-D4), hER β is expressed in greater quantities compared to hER α .

I. A human version of ER β (hER β) has also been cloned. The tissue specificity of hER β expression in a variety of cells was also determined using the

RT-PCR technique. The results are shown in Fig. 9. It will be noticed that there is a very high level of mRNA of hER β in human umbilical vein endothelial cells (HUVEC) but no detection of hER α in the same cells. In addition, it will be seen that in human osteosarcoma cell line (HOS-D4), hER β is expressed in greater quantities compared to hER α .

The partial DNA sequence of hER β is shown in Fig. 10 and a derived amino acid sequence is shown in Fig. 11.

Cloning of human Er β from testis

A commercially available cDNA from human testis (Clontech, article no. HL1161x) was screened, using a fragment containing the ligand-binding domain of the rat Er β cDNA as probe. Approximately 10⁶ recombinants were screened, resulting in one positive clone. Upon sequencing of this clone, it was seen that the insert was 1156 bp (Figure 13A and 13B). This corresponds to most of the translated region of a receptor with an overall homology of 90.0% to rat Er β , therefore deduced to represent the human form of Er β .

The cloned hER β , however, lacks approximately 47 amino acids at the N-terminal end and 61 amino acids at the C-terminal end (as compared to the rat sequence). Further screening of the same library was unsuccessful. PCR technology was therefore used to obtain the remaining parts. For

oligonucleotides were synthesised: two degenerate oligonucleotides containing all possible codons for the amino acids adjacent to the initiation methionine and the stop codon, respectively, of the rat Er β , and two specific oligonucleotides containing the sequence of the clone isolated from the human testis library and situated approximately 100 bp from respective end of this clone. PCR with the N-terminal and C-terminal pair of oligos yielded specific bands, that were subcloned and sequenced. The parts of these new clones that overlap the original cDNA clone are identical to this. It was thus possible to construct peptide and DNA sequences corresponding to the whole open reading frame (Fig. 13A and 13B).

When comparing the human Er β to rat Er β , this receptor is 79.6% identical in the N-terminal domain, 98.5% in the DNA-binding domain, 85.6% in the hinge and 91.6% in the ligand-binding and F-domains. These numbers match very well those found when comparing the rat and human forms of Er α .

Studies of the expression of human Er β using Northern blot show expression in testis and in ovaries. The expression in prostate, however, appears lower than found in the rat.

The human Er β gene has been mapped to chromosome 14 using PCR and to region 14q22-23 using the FISH technique, whereas the human Er β gene has been mapped to chromosome 6q25.

2. Comparison of ligand binding affinity of hER α and rER β

The ligand affinity of the two estrogen receptors, human Er α (ovary) (hER α) and rat Er β (rER β) was tested in binding saturation experiments and in binding competition experiments.

cDNA of the receptor subtypes hER α and rER β were *in vitro* translated in rabbit reticulocyte lysate in presence of non-radioactive amino acids according to the instructions supplied by the manufacturer (Promega).

The radioactive ligand used in all experiments was 16 α -[125 I]-17 β -estradiol ([125 I]-E2) (NEX- 144, New England Nuclear). The method for the binding experiments was previously described in: Salomonsson M, Carlsson B, Haggblad J. J. *Steroid Biochem. Molec. Biol.* Vol. 50, No. 5/6 pp. 313-18, 1994. In brief, estrogen receptors are incubated with [125 I]-E2 to equilibrium (16-18 h at +4°C). The incubation was stopped by separation of protein-bound [125 I]-E2 from free [125 I]-E2 on Sephadex G25 columns. The radioactivity of the eluate is measured in a gamma-counter.

In the competition experiments, non-radioactive ligands were diluted in DMSO, mixed with [125 I]-E2 (approximately 100-200 pM), aliquoted in

parallel, and finally hER α or rER β was added. The final concentration of DMSO in the binding buffer was 2%.

The buffer used in the experiments was of the following composition:

Hepes (pH=7.5) 20 mM, KCl 150 mM, EDTA 1 mM, glycerol (8.7%), monothioglycerol 6 mM, Na₃MO₄ 10 mM.

3. Equilibrium binding saturation experiments (K_d-determinations)

A range of concentrations of [¹²⁵I]-E2 were mixed with the ER:s and incubated as described above, free [¹²⁵I]-E2 was determined by subtracting bound [¹²⁵I]-E2 from added [¹²⁵I]-E2. Binding data was analysed by Hill-plots and by Scatchard plots (Figure 11). The equilibrium binding results are shown in Table 1. The apparent K_d-values for [¹²⁵I]-E2 differed between the two ER:s with approximately a factor of four: K_d(hER α):K_d(rER β) = 1:4.

Table 1. Equilibrium dissociation constants for [¹²⁵I]-E2 to the two subtypes.

Receptor subtype	K _d (Hill-plot)	K _d (Scatchard-plot)
hER α	0.06 nM	0.09 nM
rER β	0.24 nM	0.42 nM

4. Competition experiments (IC₅₀ determinations)

The experiments were performed as described above. IC₅₀ values were obtained by applying a four parameter logistic analysis: $b=((b_{\max}-b_{\min})/(1+(I/IC_{50})^S))+b_{\min}$, where I is the added concentration of binding inhibitor, IC₅₀ is the concentration of inhibitor at half maximal binding and S is a slope factor. The free concentration of [¹²⁵I]-E2 was determined by sampling an aliquot from the wells at the end of the incubation and then subtract bound radioactivity from sampled total radioactivity.

Since the equilibrium binding experiments (above) showed that the K_d-values for [¹²⁵I]-E2 differed between the two ER:s, K_i-values (from the Cheng-Prusoff equation: $K_i=IC_{50}/(1+L/K_d)$ where L is free (¹²⁵I)-E2) were calculated for the compounds investigated. Two approaches for calculating RBA (Relative Binding Affinity) were used. The RBA values were derived using either the IC₅₀ values or the K_i values. In both approaches, the value for the compound 16 α -bromo-estradiol was selected as the reference value (100%). Both approaches gave similar results. The results are summarized in Figure 12. In these Figures "4-OH-Tam" = 4-hydroxy-tamoxifen; "DES" = diethylstilbestrol; "Hexestr" = hexestrol; "ICI-164384" = ICI pic compound no. 164382; "17 β -E2" = 17 β -estradiol; "16a-B-E2" = 16 α -bromo-estradiol; "Ralox" = Raloxifene; and "17a-E2" = 17 α diol.

The results show that Er α and Er β have significant different ligand binding affinities - the apparent K_d-values for [¹²⁵I]-E2 differed between the two ER's by a factor of about 4 (K_d(hER α): K_d(rER β) ≈ 1:4). Some compounds investigated

showed significant differences in the competition for binding of [¹²⁵I]-E2 to the ER's. Certain compounds were found to be more potent inhibitors of [¹²⁵I]-E2 binding to hER α as compared to rER β whereas others were found to be more potent inhibitors of [¹²⁵I]-E2 binding to rER β than to hER α .

CLAIMS

1. A receptor, ER β , having the amino acid sequence of Figs. 1, 13A or 14A or substantially the same amino acid sequence as the amino acid sequence shown in Figs 1, 13A or 14A or an amino acid sequence functionally similar to those sequences.
2. A receptor according to claim 1 having an amino acid sequence which is more than 95% identical with the sequence shown in Figs. 1, 13A or 14A.
3. A receptor according to claim 1 or 2 which is derived from rat or human cells.
4. A receptor according to claim 1, 2 or 3 which is an estrogen receptor.
5. A DNA sequence encoding a receptor according to claim 1, 2, 3, or 4.
6. A DNA sequence according to claim 5 in which the DNA sequence is that given in Figs. 1, 13B or 14B or is a DNA sequence encoding a protein or polypeptide having the functionality of ER β .
7. The use of a receptor according to claim 1, 2 ,3 or 4 or a DNA sequence according to claim 5 or 6 in an assay to determine molecules which bind Er β .
8. The use of a receptor according to claim 1, 2, 3 or 4 or a DNA sequence according to claim 5 or 6 in an assay to determine molecules for use in the treatment of Er α or Er β specific diseases or conditions.

9. The use of a receptor according to claim 1, 2, 3 or 4 or a DNA sequence according to claim 5 or 6 in an assay to determine molecules for use in treatment of prostate or ovarian cancer, benign prostatic hyperplasia, diseases of the central nervous system, osteoporosis, or cardiovascular disease.
10. A drug design method comprising comparing binding of a test compound to ER α and to ER β .
11. hER β and functional equivalents thereof.
12. The use of a receptor according to claim 1, 2, 3 or 4 in the testing of the possible estrogenic or other hormonal effect of a substance.

FIG. 1

1/17

G C A B I I C G G G G G A G C T G G G C C A G G G G G G C T G G C A C T G G C A T C C T A G G C 60
 A C C O A B S T C T G C A A T A A A G T C T G G C A G C C A C T G C A T G G C T G A G G C A C A A C C A G T G G C T G G 120
 G A G T T C C G G C T C T G T G G C T G A G G A A A G C A C C T G T C T G C A T T T A G A G A A T G C A A A A T A G A G A 180
 A T G T T T A C C T G C C A G T C A T T A C A T C T G A G T C C C A T G A G T C T C T G A G A A C A T A A T G T C C H T 240
 C T G T A C C T C T T C T C A C A A G G A G T T T C T C A G G C T G C G A C C C T C T G A A G A C A T G G A G A T C A A 300
 A A R C T C A C C G T G A G G C T T A G T T C C T G C T T C T A T A A C T G T A G G C C A G T C C A T C C T A C C C 360
 C T G G A G C A C G G G C C C A T C A C A T C C C T C C T C C T A C G T A G A C A C C G C C A T G A C T A T T C A 420
 G C T A T G A C A T T C A C A G T C C T G C T G A T G A A C T A C A G T G T C C C G C A G C A C C A G T A A C 480
 M T P Y S P A V M N Y S V P G S T S X
 C T G G A C G G T G G G C C T G T C C A C T G A G G C A C A A G C C C A A A T G T G C T A T G G C C A C T T C T G G G 540
 I D G G P V F L S T S P N V L W P T S G
 C A C C T G T C T C T T T A G C G A C C C A T T G C C A A T C A T C G C T C C T C T A T G C A G A A C C T C A A A A G 600
 H I S P L A T H C Q C S S L L Y A E P Q K
 A G T C T T T G G T G A R S C A A G A T C A C T A G G C A C A C C T T A C C T G T A A A C A G A G A G A C A C T G 660
 S P W C E A R S L E H T I P V N R E F L
 A A G A G G A A G C T T A G T G G G A G C A G T T G G C C A G G C C T G T T A C T A G T C C A A A C G C A A A G A G G 720
 H P H I S G S S C A S P V T S P N A K F
 G A T G C T C A C T T C T G C C C G T C T G C A G G C A T T A T G C A T C T G G G T A T C A T T A C G G C G T T T G G 780
 D A H F C P V C S D Y A S G Y H Y G V W
 T C A T G T G A A G G A T G T A A G G C C T T T T T T A A A A G A G C A T T C A A G G A C A T A A T G A T T A T A T C 840
 S I E G S H A F F R P S I Q G H N D Y I
 T G T C C A G C C A C C G A A T C A G T G T A C C A T A G A C A A G A A C C G G C G T A A A G C T G C C A G G C C T G G 900
 C P A T M C G T I D K N F P H S C Q A C
 C G A C T T C G G A A C T G T T A T G A A G T A G G A A T G G T C A A S T G T G G A T C C A G G A G A G A A C G G T G T 960
 F I P H C Y E V G M V K C G S R A F E R C
 G G G T A C C G T T A T A G T G C G G A G G C A G G A A G T T C T A G G C A G C A G G T A C A C T G C C T G A G G C A A 1020
 G Y P I V R R Q P R S S S E Q V H C L S K
 G C C A A G A G A A A C C G G T G G G C A T G C A C C C C C G G G T G A A G G A G C T A C T G C T G A G C A C C T T G A G T 1080
 A H P N G G H A P R V K E L L L S T L S
 C C A G A G C A A C T G G T G C T C A C C S T C T G G A A G C T G A A C C A C C C A A T G T G C T G G T G A G C C G T 1140
 P E Q I V L T L L E A E P P N V L V S F
 C C C A G G C A T G C C C C T C A C C G A G G C C T C C A T G A T G A T G T C C C T C A C T A A G C T G G C G G A C A A G 1200
 P S M P F T E A S M M M S L T K L A D H
 G A A C T G G T G C A C A T G A T T G G C T G G G C C A A G G A A A A T C C C T G G C T T G T G G A G G C T C A G G C C T G 1260
 E I V H M I G W A K K I P G F V E L S L
 T T G G A C C A A G T C C G G C T C T T A G A A A G C T G C T G G A T G G A G G T G C T A T G G T G G G A C T G A T G 1320
 I D Q V R L L E S C W M E V L M V G L M
 T G G C C T C C A T G A C C A C C C C C G G C A A G G T C A T T T C G C T C C C G A C C T C G T T C T G G A C A G G 1380
 W P S I D H P G K I F A P D L V L D F
 G A T G A G G G G A A G T C C G T A G A A G G G A T T C T G G A A A T T T T G A C A T G C T C T G G C G A C G A C G 1440
 D E G H C V E G I L E I F D M L L A T I
 T C A A G S T T C C G T G A G T T A A A A C T C C A G C A C A A G G A G T A T C T C T G T G T G A A G G C C A T G A T C 1500
 S P F F E I H L Q H K E Y I C V K A M I
 C T C C T C A A C T C C A G T A T G T A C C C C T T G G C T T C T G C A A A C C A C C A G G A G G C A G A A A G T A G C C G G 1560
 L I H S S M Y P L A S A N Q E A E S S P
 A A G C T G A C A C A C C T A C T G A A C C G C G T G A C A G A G T G C C C T G G G T G A T T G C G A A G A G T 1620
 K I T H L I N A V T D A L V W V I A K S
 G G T A T C T C T C C C A G C A G C A G T C A G T C C G A C T G G C C A A C C T C C T G A T G C T T C T T T C T C A C 1680
 G I S S C Q S V R L A N L I M L L S H
 G T C A G G C A C A T C A G T A A C A A G G G C A T G G A A C A T C T G C T C A G G C A T G A A G T G C A A A A A T G T G 1740
 H F H I S N H G M E H L L D S M K C K N V
 G T C C C G G T G T A T G A C C T G C T G T G G A G A G T G C T G A T G C T C A C C G C T T C G A G G G T A C A A G 1800
 W P V Y D L L D E M I N A H C L D R G Y K
 C C C T C A A T C T C G G G G T C T G A G T G C A G G T C A A C A G A G G G A C A G T A A C A A A A G A G A G G C T C 1860
 S S I S G S E C S S T E D S H N H E S S
 C A G A A C C T A C A G T C T C A G T G A T G G C C A G G C C T G A G G C G G G A C A G A C T A C A G A G A T G G T C A A 1920
 C N I C S C
 A A G T G G A A C A T G T A C C C T A G C A T C T G G G G G T T C C T C T T A G G G C T G C C T T G G G T T A C G C A C C 1980
 C C T T A C C C A C A C T G C A C T T C C A G G A G T C A G G G G T G G G T G T G C C G G T G T T C C T C A T A C C 2040
 A G G A T G T A C C A C C G A A T G C C A A G T T C T A C T T G T A T A G G C C T T G A A G G C T C T C G G T G T A C T 2100
 T A C T T T C T G T C C T T G C C C A C T T G G A A A C A T C T G A A A G G T T C T G G A A C T A A A G G T C A A A 2160
 G T C T G A T T T G G A A G G A T T G T C T T T A G G C A G G A A A A G G A A T A T G C C A T G T G A C A C A G C T A T 2220
 A A G A A T G G A C T G T A G G A C T G T G G C C C T A A A A T C A A C C T T G G A T T G G C G T T T C T A G A 2280
 C C A C T T G A T T G T A G G A T T G A A A A C C A C A T T G A C A A T C G C T C A T T T C G C A T T C T G C C T C 2340
 A C G G G T C T G T G A G G A C T C A T T A A T G T C A T G G G T T A T T C T A T C A A A G A C C A G A A A G A T A G T 2400
 G C A A G C T T A G A T G T A C C T T G T G C C C T C C C A G A C C C T T G G G T T A C T C T T A G A G G C C T S 2460
 C T T A T T T G G T C T G T G A A T G T G G T C A T T G T C A T G G G T T A A G A T T T A A T C T C T T T G T A A 2520
 T A T T G G T C T T G A A G C T A T G T C A T T T T C T C T C T C C C G g a a t i c 2580

SUBSTITUTE SHEET (RULE 26)

2 / 17

FIG. 2A

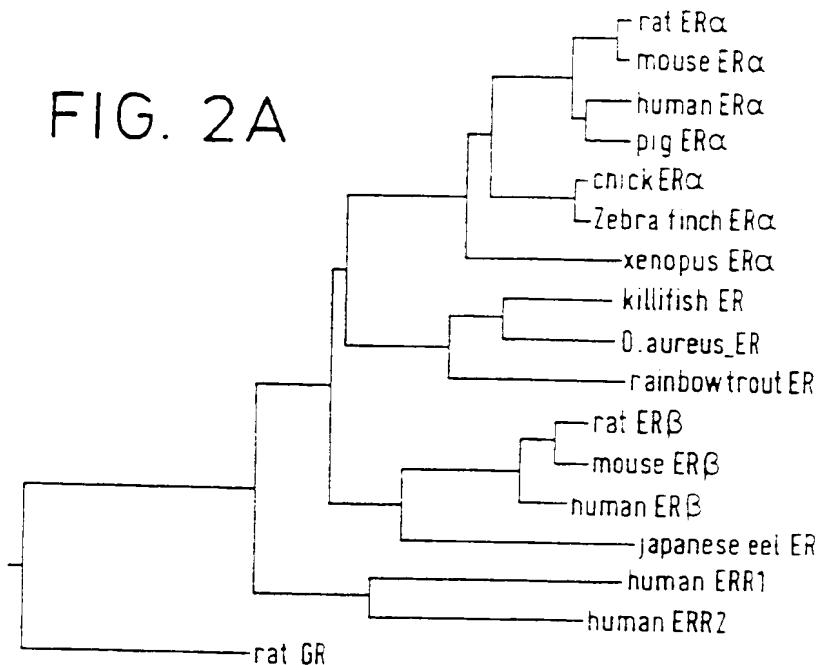


FIG. 2B

ALIGNMENT OF ER β TO OTHER
ESTROGEN RECEPTORS

A/B	DED	D	E/F	rat ER β
79.6	98.5	85.6	91.6	human ER β
89.3	97.0	92.2	95.6	mouse ER β
16.5	95.5	28.9	53.5	rat ER α
17.5	95.5	28.9	54.4	human ER α
16.5	69.7	15.6	34.2	human ERR1
13.7	72.7	19.8	31.0	human ERR2

SUBSTITUTE SHEET (RULE 26)

3 / 17

Ligandbinding domain

SUBSTITUTE SHEET (RULE 26)

FIG. 2C

4 / 17

DNA-binding domain

	C P V C S D Y A S G Y H Y G V W S C E G C K A F F K R A S I Q G H N D Y I C P A T N G C T I D K N R R K S C O A C R L R K C Y E V G M	ER01m
A	N	ERrat
A	N	ERmouse
A	N	ERhuman

FIG. 2D**SUBSTITUTE SHEET (RULE 26)**

5 / 17



FIG. 3A



FIG. 3B

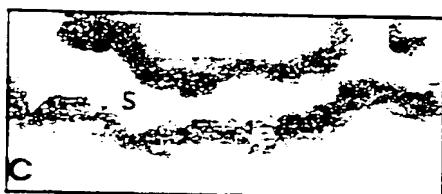


FIG. 3C

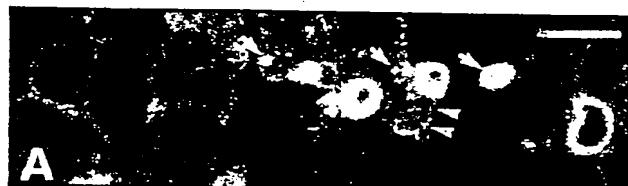


FIG. 4A

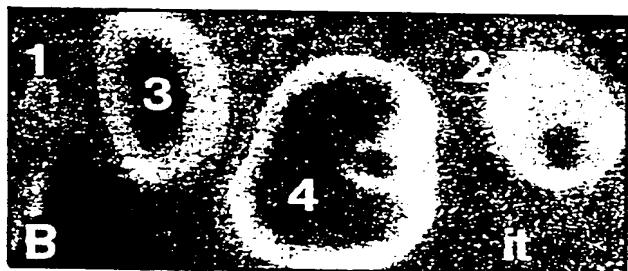


FIG. 4B



FIG. 4C

SUBSTITUTE SHEET (RULE 26)

6/17

SCATCHARD PLOT OF ER-BETA

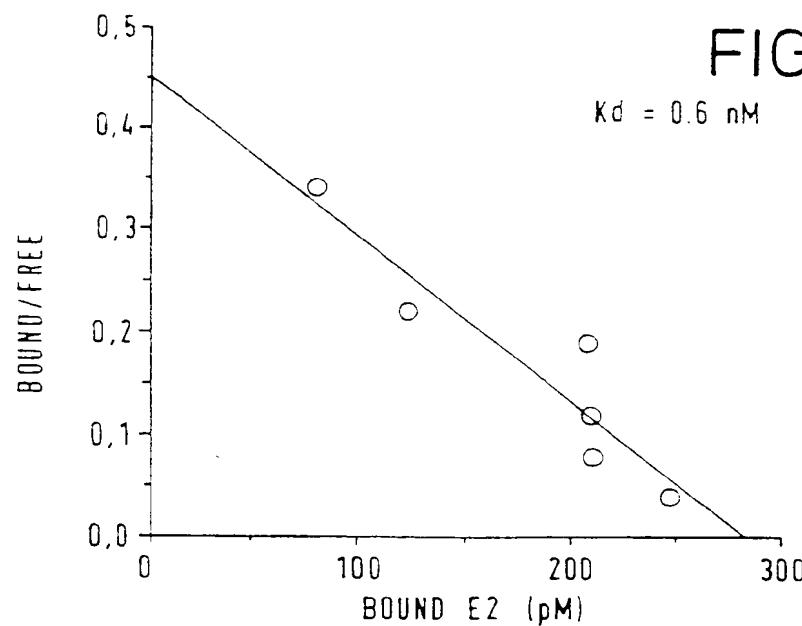
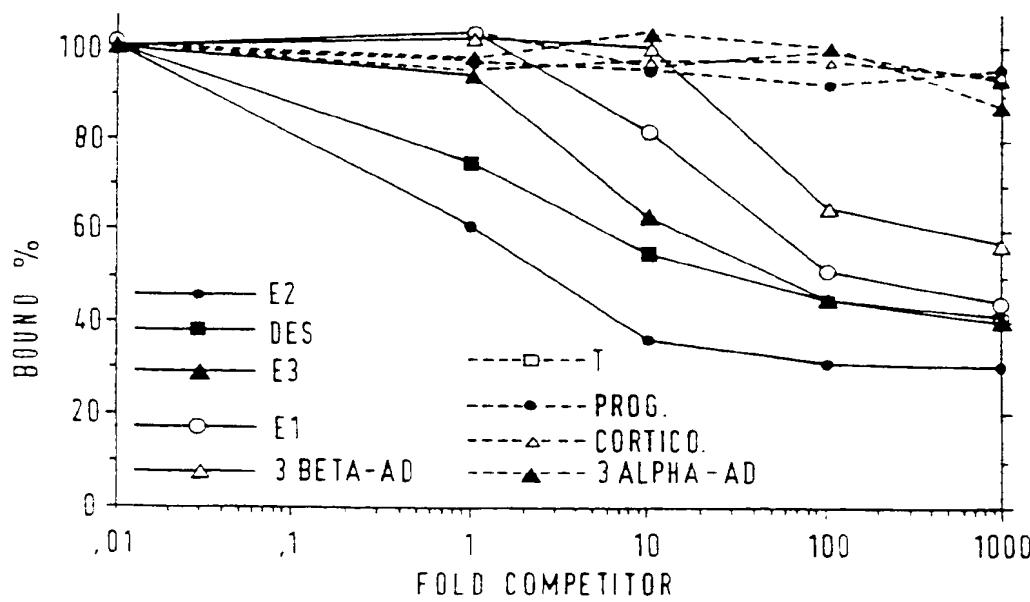


FIG. 5A

$K_d = 0,6 \text{ nM}$

ER-BETA LIGAND SPECIFICITY

FIG. 5B



SUBSTITUTE SHEET (RULE 26)

7/17

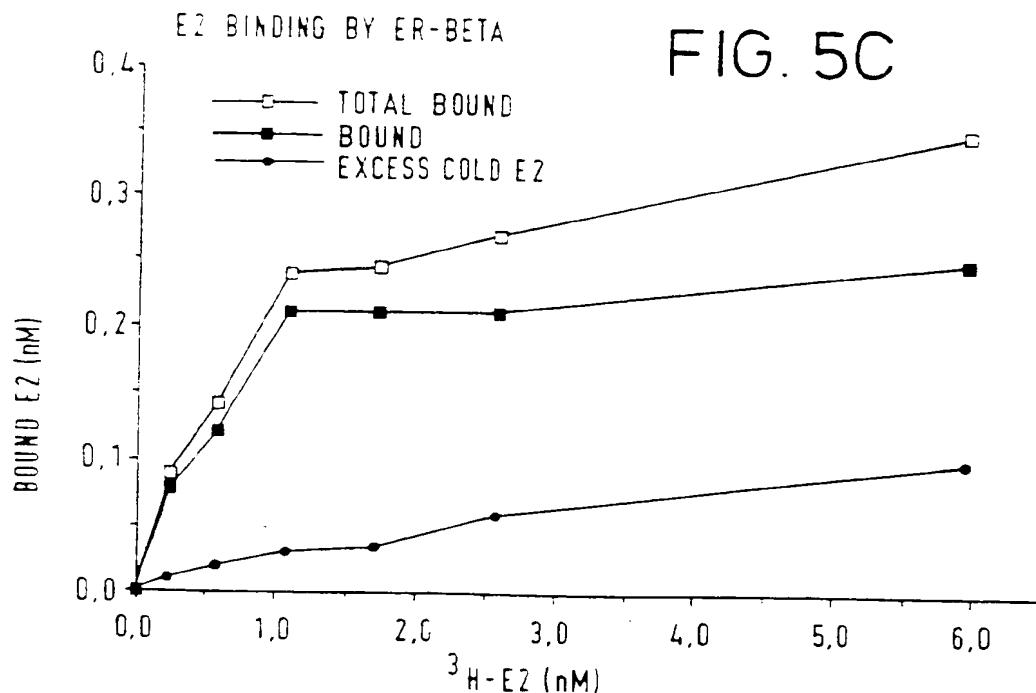
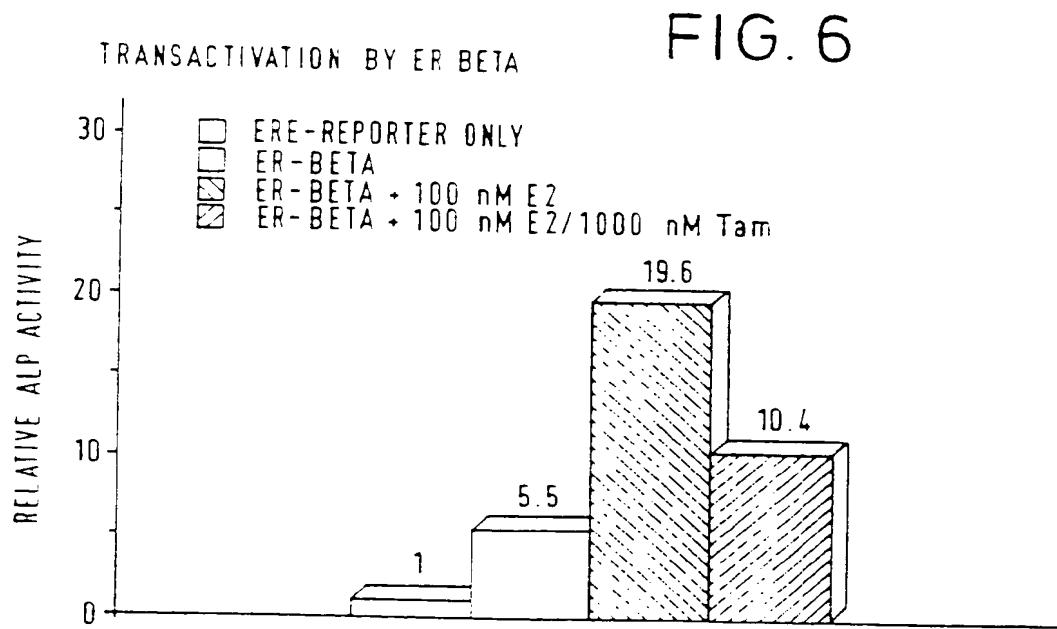


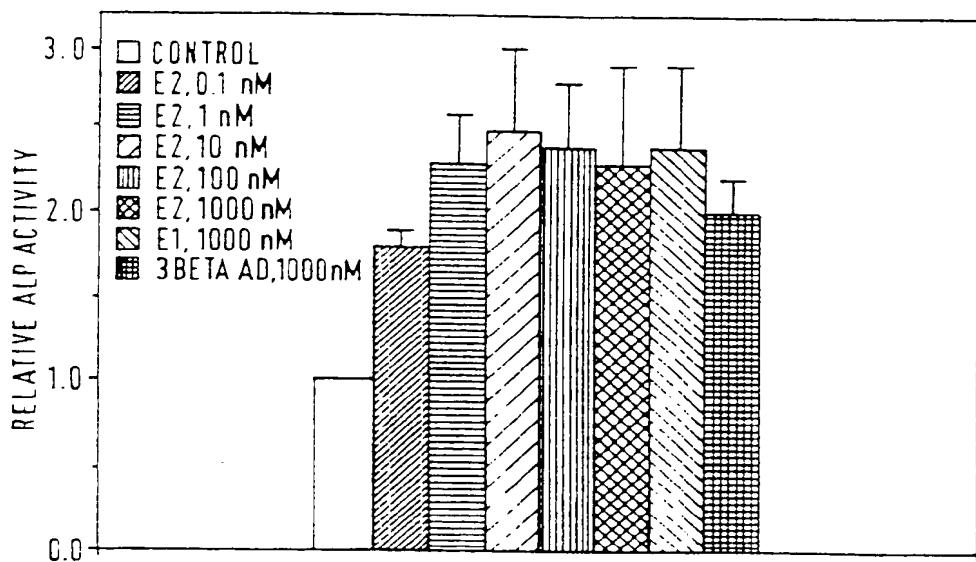
FIG. 5C



SUBSTITUTE SHEET (RULE 26)

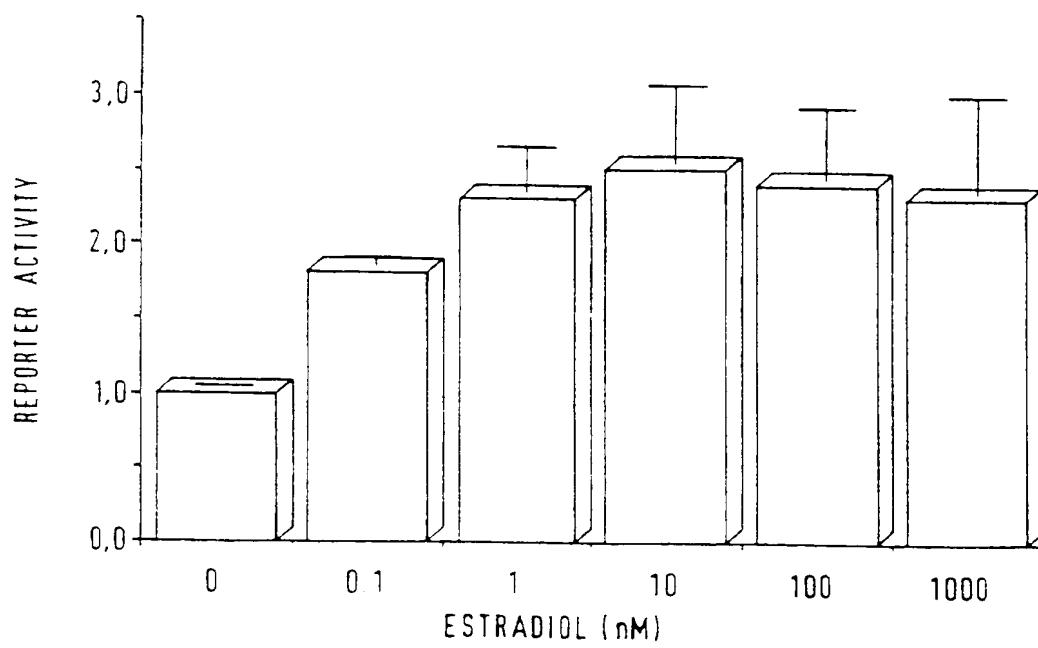
8 / 17

FIG. 7



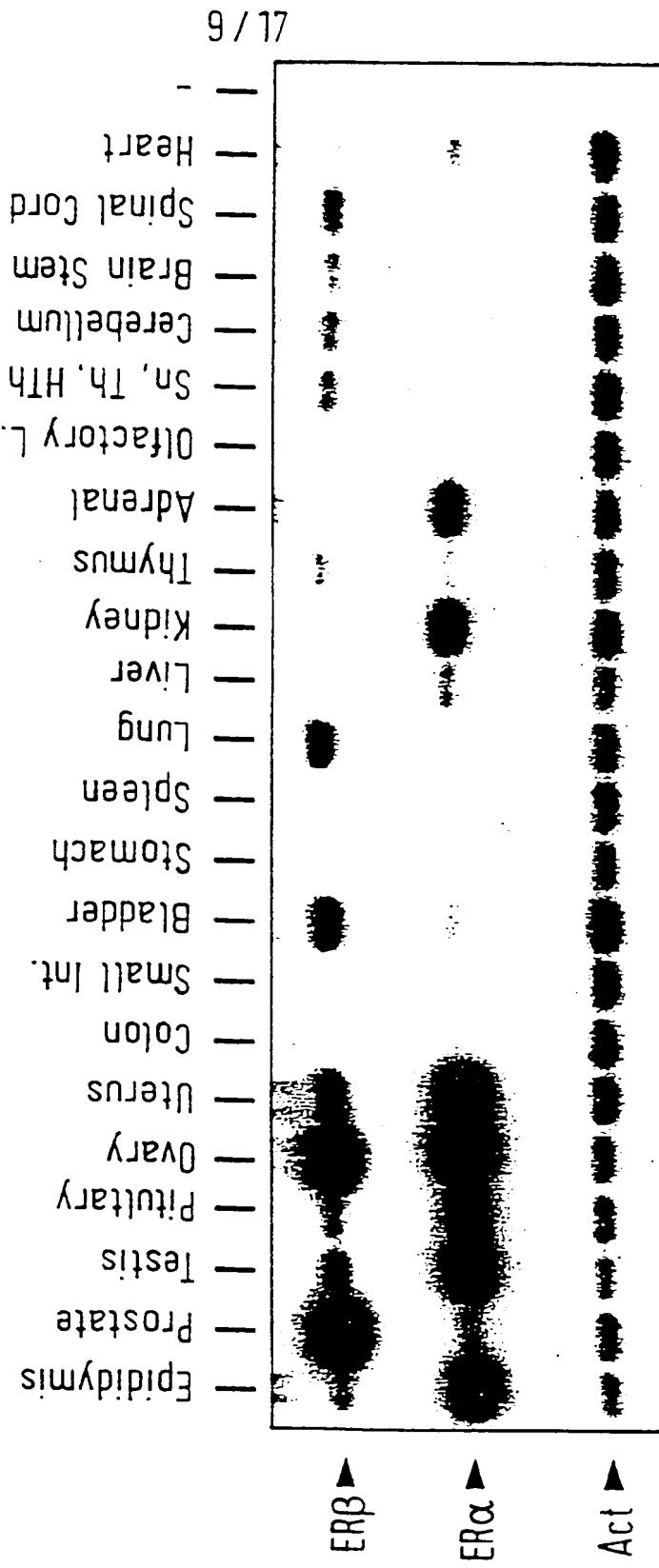
E2 STIMULATED TRANSACTIVATION

FIG. 7A



SUBSTITUTE SHEET (RULE 26)

DETECTION OF RAT ER EXPRESSION BY RT-PCR.



SUBSTITUTE SHEET (RULE 26)

FIG. 8

10 / 17

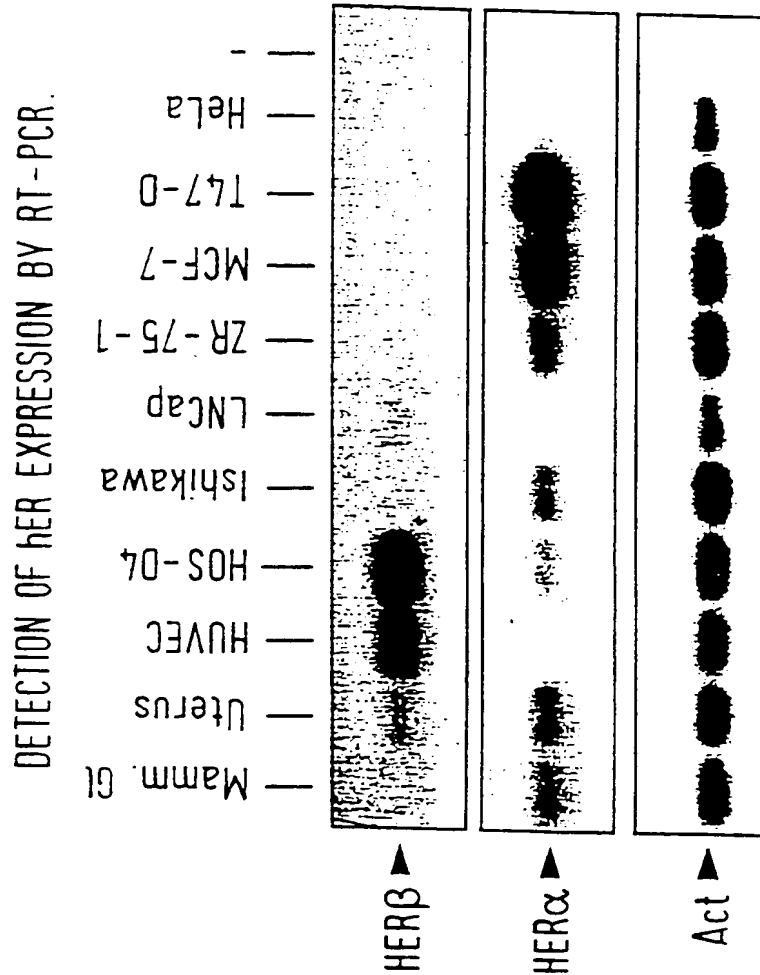


FIG. 9

SUBSTITUTE SHEET (RULE 26)

FIG. 10A

11/17

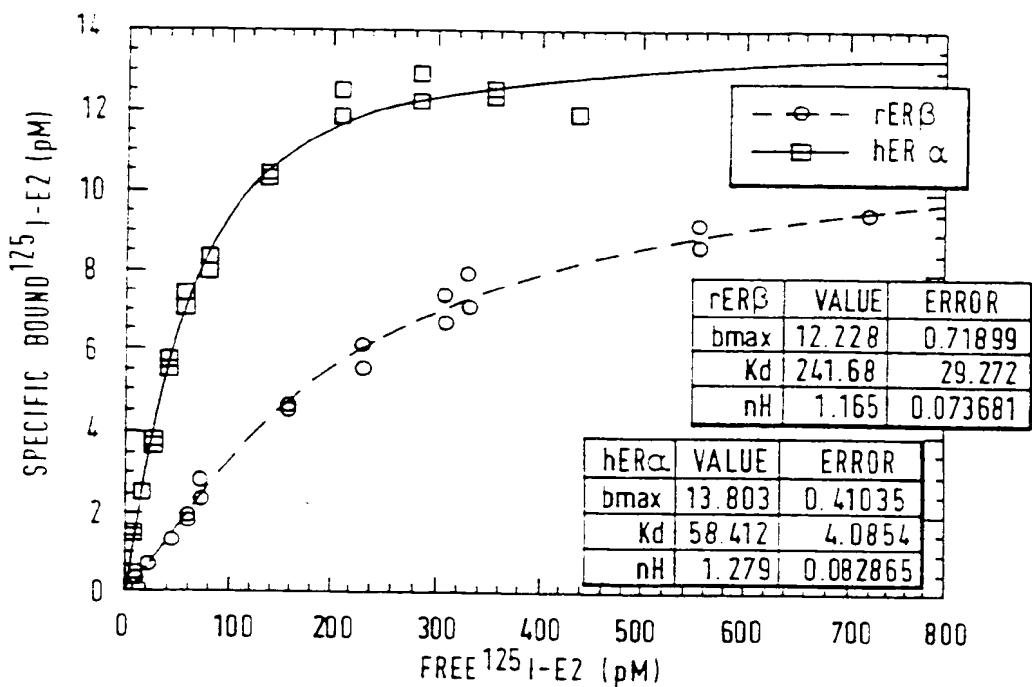
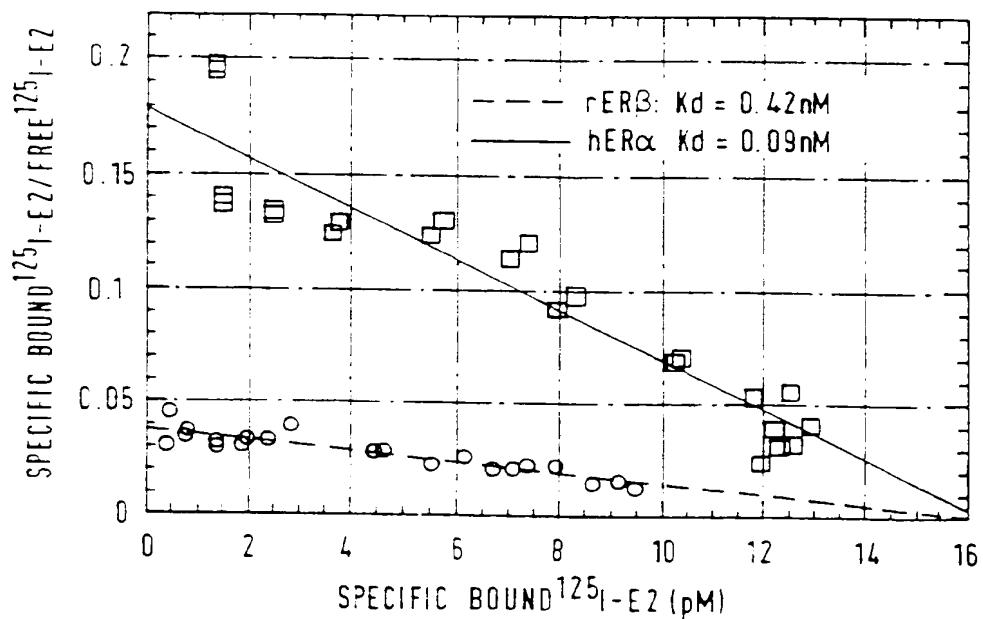
HILL PLOT COMPARING hER α AND rER β .

FIG. 10B

SCATCHARD PLOT COMPARING hER α AND rER β .

SUBSTITUTE SHEET (RULE 26)

FIG.11A

12 / 17

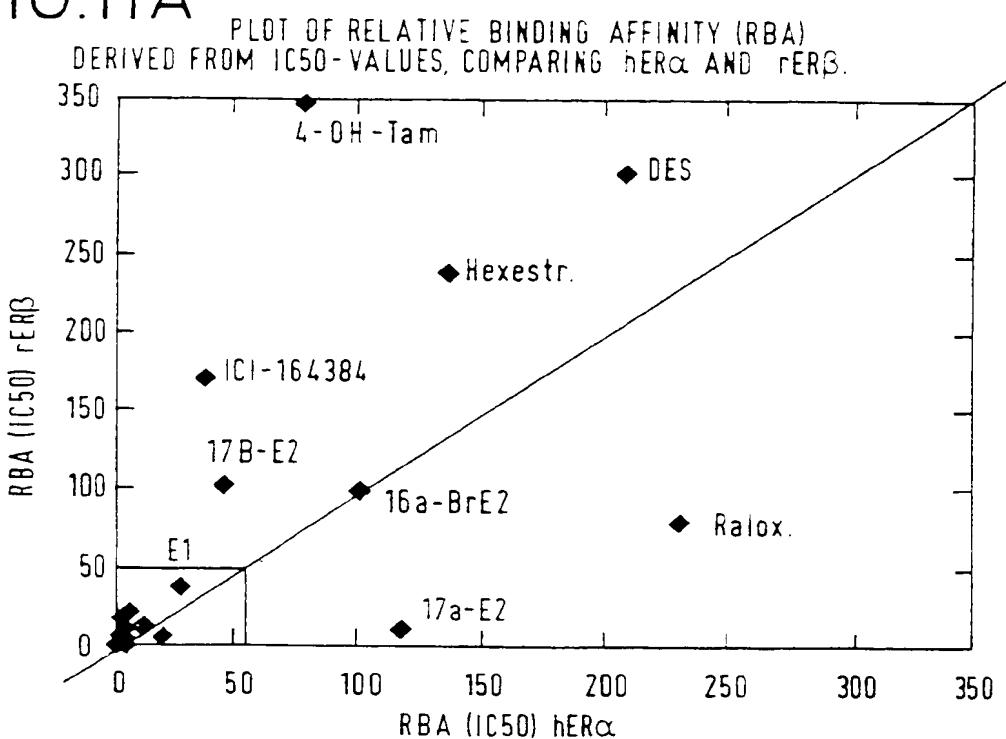
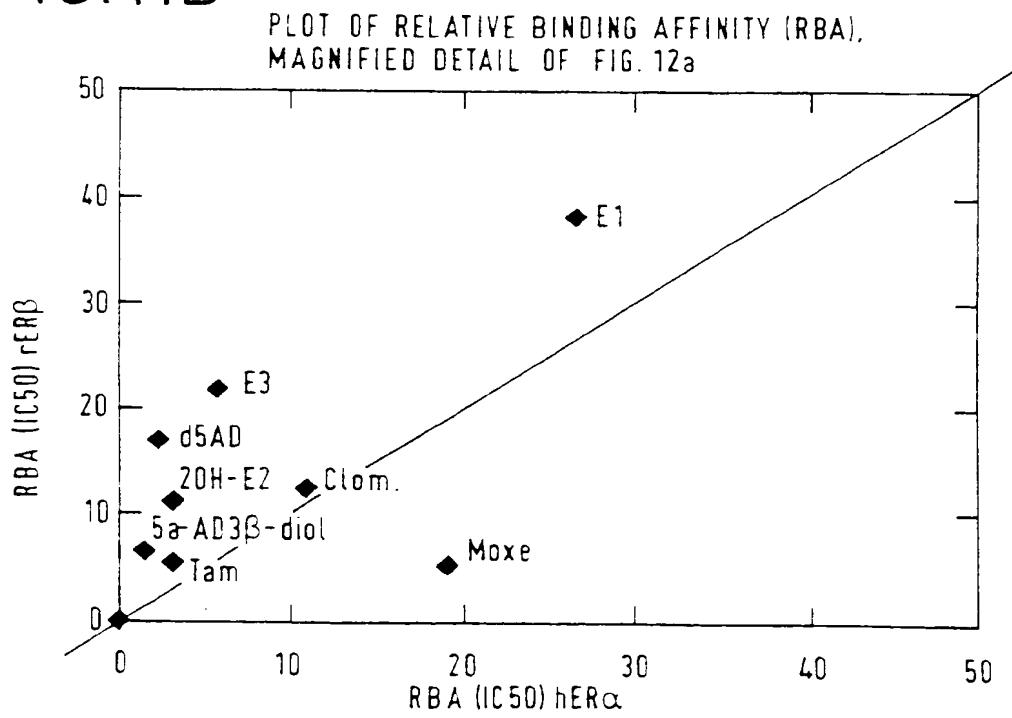


FIG.11B



SUBSTITUTE SHEET (RULE 26)

13 / 17

FIG. 12

Aligned Report of 116, p. 1 (n. 1) of Yang Chih-chia method with I&M¹⁰ resideo weight table

SUBSTITUTE SHEET (RULE 26)

14 / 17

1 MTFYSPAVMN YSIPSNVTNL EGGPGRQTT S PNVLPPTPGH LSPLVVHRQL
 51 SHLYAEPQKS PWCEARSLEH TLPVNRETLK RKVSGNRCAS PVTGPGSKRD
 101 AHFCAVCSDY ASGYHYGVWS CEGCKAFFKR SIQGHNDYIC PATNQCTIDK
 151 NRRKSCQACR LRKCYEVGMV KCGSRRERCG YRLVRRQRSA DEQLHCAGKA
 201 KRSGGHAPRV RELLLDALSP EQLVLTLEA EPPHVLISRP SAPFTEASMM
 251 MSLTKLADKE LVHMISWAKK IPGFVELSLF DQVRLL ESCW MEVLMGGLMW
 301 RSIDHPGKLI FAPDLVLD RD EGKCV E G I L E IFDMLLATT S RFRELKLQHK
 351 EYLCVKAMIL LNSSMYPLVT ATQDADSSRK LAHLLNAVTD ALVWVIAKSG
 401 ISSQQQSMRL ANLLMILLSHV RHASNKGMEH LLNMKCKNVV PVYDLLLEM
 451 NAHVLRGCKS SITGSECSPA EDSKSKEGSQ NLQSQ*

FIG. 13A

MAFYSPAVMNYSVPSSTGNLEGGPVROTA SPNVLWPTSGH 40
 LSPLATHCQSSLLYAE PQKS PWCEARSLEH TLPVNRETLK 80
 RKLGGSGCASPVTSPSTKRD AHFCAVCSDY ASGYHYGVWS 120
 CEGCKAFFKR SIQGHNDYIC PATNQCTIDK NRRKNCQACR 160
 LRKCYEVGMVKGSRERCGYRIVRRQRSASEQVHCLNKA 200
 KRTSGHTPRVKELLNSLSPEQLVLTLEAEPPNVLVSRP 240
 SMPFTEASMMMSLTKLADKELVHMIGWAKKIPGFVELSLL 280
 DQVRLL ESCWMEVLMVGLMWRSIDHPGKLIFAPDLVLD RD 320
 EGKCV E G I L E IFDMLLATTARFRELKLQHKEYLCVKAMIL 360
 LNSSMYHLATASQEAE SSRKLT HLLNAVTDALVWVISKS R 400
 ISSQQQSVRLANLLMILLSHVRHISNKGM E HLLSMKCKNVV 440
 PVY DLLLEMILNAHTLRGYKSSISGSGCCSTEDSKSKEGSQ 480
 NLQSQ. 486

FIG. 14A

SUBSTITUTE SHEET (RULE 26)

15 / 17

1	CTATGACATT	CTACAGTCCT	GCTGTGATGA	ATTACAGCAT	TCCCAGCAAT
51	GTCACTAACT	TGGAAGGTGG	GCCTGGTCGG	CAGACCACAA	GCCCAAATGT
101	GTTGTGGCCA	ACACCTGGGC	ACCTTCTCC	TTTAGTGGTC	CATGCCAGT
151	TATCACATCT	GTATGCGGAA	CCTCAAAAGA	GTCCTGGTG	TGAAGCAAGA
201	TCGCTAGAAC	ACACCTTAC	TGTAAACAGA	GAGACACTGA	AAAGGAAGGT
251	TAGTGGGAAC	CGTTGCGCCA	GCCCTGTTAC	TGGTCCAGGT	TCAAAGAGGG
301	ATGCTCACTT	CTGCGCTGTC	TGCAGCGATT	ACGCATCGGG	ATATCACTAT
351	GGAGTCTGGT	CGTGTGAAGG	ATGTAAGGCC	TTTTTTAAAAA	GAAGCATTCA
401	AGGACATAAT	GATTATATT	GTCCAGCTAC	AAATCAGTGT	ACAATCGATA
451	AAAACCAGGCG	CAAGAGCTGC	CAGGCCTGCC	GACTTCGGAA	GTGTTACGAA
501	GTGGGAATGG	TGAAGTGTGG	CTCCCCGGAGA	GAGAGATGTG	GGTACCGCCT
551	TGTGCGGAGA	CAGAGAAGTG	CCGACGAGCA	GCTGCACTGT	GCCGGCAAGG
601	CCAAGAGAAC	TGGCGGCCAC	GCGCCCCGAG	TGCGGGAGCT	GCTGCTGGAC
651	GCCCTGAGCC	CCGAGCAGCT	AGTGCTCACC	CTCCTGGAGG	CTGAGCCGCC
701	CCATGTGCTG	ATCAGCCGCC	CCAGTGCAGCC	CTTCACCGAG	GCCTCCATGA
751	TGATGTCCCT	GACCAAGTTG	GCCGACAAGG	AGTTGGTACA	CATGATCAGC
801	TGGGCCAAGA	AGATTCCCAG	CTTTGTGGAG	CTCAGCCTGT	TCGACCAAGT
851	CGGGCTCTTG	GAGAGCTGTT	GGATGGAGGT	GTTAATGATG	GGGCTGATGT
901	GGCGCTCAAT	TGACCACCCC	GGCAAGCTCA	TCTTTGCTCC	AGATCTTGT
951	CTGGACAGGG	ATGAGGGAA	ATGCGTAGAA	GGAATTCTGG	AAATCTTGA
1001	CATGCTCCTG	GCAACTACTT	CAAGGTTTCG	AGAGTTAAAAA	CTCCAACACA
1051	AAGAATATCT	CTGTGTCAAG	GCCATGATCC	TGCTCAATT	CAGTATGTAC
1101	CCTCTGGTCA	CAGCGACCCA	GGATGCTGAC	AGCAGCCGGA	AGCTGGCTCA
1151	CTTGCTGAAC	GCCGTGACCG	ATGCTTTGGT	TGGGGTGATT	GCCAAGAGCG
1201	GCATCTCCTC	CCAGCAGCAA	TCCATGCGCC	TGGCTAACCT	CCTGATGCTC
1251	CTGTCCCACG	TCAGGCATGC	GAGTAACAAG	GGCATGGAAC	ATCTGCTCAA
1301	CATGAAGTGC	AAAAATGTGG	TCCCAGTGT	TGACCTGCTG	CTGGAGATGC
1351	TGAATGCCCA	CGTGCTTCGC	GGGTGCAAGT	CCTCCATCAC	GGGGTCCGAG
1401	TGCAGCCCCG	CAGAGGACAG	AAAAAGCAAA	GAGGGCTCCC	AGAACCTACA
1451	GTCTCAGTGA				

FIG. 13B

SUBSTITUTE SHEET (RULE 26)

16 / 17

ATGGCATTCTAC	AGTAACTACAGT	ATGAACTACAGT	GTTCCAGCAGC	ACCGGTAAACCTG	GAAGGTTGGCCT	72
GTTGCCAGACT	GCAAGCCCCAAT	GTCGCTATGGCCA	ACTTCCTGACAC	CTCTCTCCCTTA	GCCACCCACTGC	144
CAATCATCGCTT	CTCTATGCGAA	CCTCAAAAGAGT	CCTGGGTGTGAA	GCAAGATCACTA	GAACACACTCTTG	216
CCTGTAACAGA	GAGACCTCTGAAG	AGGAAGCTTGGC	GGAGAGGGTTGT	GCCAGGCCCTGTT	ACTAGTCCAAAGC	288
ACCAAAGGGAT	GCTCACTTCTGT	GCCGTCCTGCAGT	GATTATGCATCT	GGGTATCATTAC	GTTGTCCTGTC	360
TGTGAAGGATGT	AAGGCCCTTTTT	AAAAGAACCTT	CAAGGACATAAT	GACTATATCTGT	CGGCCACGAAT	432
CAGTGTACGATA	GACAAGAACCG9	CGTAAAAAACTGC	CAGGCCCTGCCGA	CTTCGCAAGTGT	TACGAAGTAGGA	504
ATGGTCAAGTGT	GGATCCAGGAGA	GAAAGGTGTTGGG	TACCGAATAGTA	CGAAGACAGAGA	AGTGGCCAGGGAG	576
CAGGTGCAATTGC	CTGAAACAAAGCC	AAGAGAACCCAGT	GGGCACACACCC	CGGGTGAAGGAG	CTACTGGTGNAC	648
TCTCTGAGTCCC	GAGCAGCTGGTG	CTCACCCCTGCTG	GAAGCTGAGCCA	CCCAATGTGCTA	GTTGAGTCTGCCC	720
ACCGAGGCCCTTC	ACCGAGGCCCTCC	ATGATGATGTCC	CTTACGAAAGCTG	GCTGACAAAGGAA	CTGGGTGCACATG	792
ATTGGCTGGCC	AAGAAAATCCCT	GGCTTGTGGAG	CTCAGCCTGTTG	GACCAAGTCCGC	CTCTTGGAAAGC	864
TGCTGGATGGAG	GTGGCTGATGGTG	GGGCTGATGTGG	CGCTCCATCGAC	CACCCCGGCAAG	CTCATCTTGCT	936
CCAGACCTCGTT	CTGGACAGGGAT	GAGGGAAAGTGC	GTggAAAGGGATT	CTGGAAATCTTT	GACATGCTCCTG	1008
GCGACGACGGCA	CGGTTCGGTAC	TTAAAACACTGCG	CACAAAGAATAT	CTGTGTTGAAAG	GCCATGATCTC	1080
CTCAACTCCAGT	ATGTAACCACTTG	GCTACCGCAAGC	CAGGAAGCAGAG	AGTAGCCGGAAG	CTGACACACCTA	1152
TTGAAACGGCAGTG	ACAGATGCCCTG	GTCTGGGTGATT	TCGAAGAGTGA	ATCTCTTCCCAG	CAGCAGTCAGTC	1224
CGTCTGGCCAAAC	CTCCCTGATGCTT	CTTtCTCATGTC	AGGCACATCAGT	AACaaAGGCATG	GAACATCTGCTC	1296
AGCATGAAGTGC	GGGTACAAGTCC	AAAAATGTGGTC	CGGTGTTACGAC	ATGCTGATGCT	CACACGGCTCGA	1368
AACTCCAGTCT	TCAATCTGGGG	TCTGGGTGCTGC	TCCGACAGAGGAC	AGTAAGAGCAA	GAGGGCTCCAG	1440
	CAGTGA	1458				

FIG. 14B

SUBSTITUTE SHEET (RULE 26)

17 / 17

CPO	log IC ₅₀ hER _{RA}	log IC ₅₀ hER _{RA}	[IC ₅₀ (nM)] hER _{RA}	[IC ₅₀ (nM)] hER _{RA}	K _i (nM) hER _{RA}	RBA(%). K _i hER _{RA}	RBA(%). K _i hER _{RA}	RBA(%). K _i hER _{RA}
Dihydroandrostenedione	-6.31	-6.73	485.29	187.11	245.31	163.33	0.027	0.115
Testosterone	-5.00	-5.66	10000.00	2187.76	5750.97	1937.70	0.001	0.010
Dihydrotestosterone	-6.36	-7.08	436.52	83.95	220.66	73.28	0.030	0.256
4-OH-Fstradiol	-8.78	-8.67	1.66	2.14	0.95	1.89	6.934	7.889
19-Nor-testosterone	-5.82	-7.22	1513.56	60.12	765.10	52.48	0.009	0.357
5 β -Androstanedione	>4	>4	>100000	>100000	>100000	>100000	<0.00012%	<0.0002%
Cyproteroneacetate	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
5 α -androstene 3,17-dione	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
Progesterone	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
Conicosterone	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
Gentistein	-8.35	-9.41	4.47	0.39	2.57	0.34	2.576	54.361
β -sitosterol	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
norethynodrel	-7.53	-7.20	29.51	63.10	14.22	53.09	0.466	0.353
norethindrone	-6.50	-5.89	316.23	1288.25	152.32	1083.89	0.043	0.017
β -nearanol	-8.89	-9.00	1.29	0.99	0.78	0.87	8.457	21.465
D ₄ -andosten 3 β ,17 β -diol	-7.33	-7.64	46.77	22.91	23.39	18.71	0.283	1.001
dienestrol	-10.03	-10.46	0.09	0.03	0.05	0.03	140.523	661.418
Methoxychlor	-5.45	-6.96	3548.13	109.65	1774.07	89.56	0.004	0.209
Bisphenol A	-6.41	-7.37	389.05	42.66	194.52	34.84	0.034	0.538
Ecdysterone	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
Eudesmine	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
Lapidine	>4	>4	>100000	>100000	>100000	>100000	<0.0002%	<0.0002%
Tschimganidine	-7.18	-6.63	66.07	234.42	40.13	206.47	0.165	0.091
Ferulinine	-7.87	-6.45	13.49	354.81	8.19	312.50	0.808	0.060
Coumestrol	-9.10	-9.56	0.79	0.27	0.40	0.24	16.623	78.091
Naloxidine	-9.65	-10.12	0.22	0.08	0.14	0.07	48.665	282.307
16 α -Bi-E2	-9.32	-9.05	0.48	0.90	0.24	0.78	27.530	23.966
17 α -E2	-9.88	-9.67	0.13	0.21	0.07	0.19	100.000	100.000
17 β -E2	-9.44	-8.88	0.36	1.32	0.22	1.16	30.006	16.133
	-9.68	-9.87	0.21	0.13	0.12	0.12	52.145	157.660
							62.661	62.661
							159.074	159.074
β-Ecdysone, 20-Hydroxyecdysone RBA values derived from 16α-Br-E2 (100%)								

FIG. 15

SUBSTITUTE SHEET (RULE 26)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

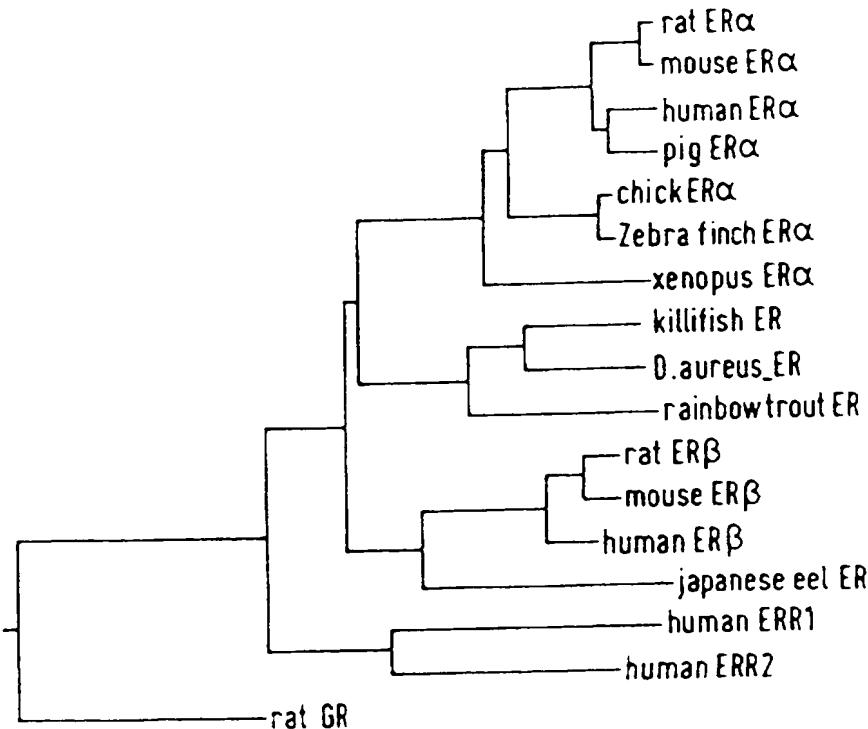
INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, C12Q 1/68, C07K 14/705		A3	(11) International Publication Number: WO 97/09348 (43) International Publication Date: 13 March 1997 (13.03.97)
(21) International Application Number: PCT/EP96/03933		(81) Designated States: AU, CA, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(22) International Filing Date: 9 September 1996 (09.09.96)			
(30) Priority Data: 9518272.1 8 September 1995 (08.09.95) GB 9605550.4 15 March 1996 (15.03.96) GB 9607532.0 11 April 1996 (11.04.96) GB 9609576.5 8 May 1996 (08.05.96) GB			
(71) Applicant (for all designated States except US): KARO BIO AB [SE/SE]; Novum, S-141 57 Huddinge (SE).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendment(s).</i>	
(72) Inventors; and		(88) Date of publication of the international search report: 24 April 1997 (24.04.97)	
(75) Inventors/Applicants (for US only): KUIPER, George, G., J., M. [SE/SE]; Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Novum, S-141 86 Huddinge (SE). ENMARK, Eva [SE/SE]; Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Novum, S-141 86 Huddinge (SE). GUSTAFSSON, Jan-Ake [SE/SE]; Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Novum, S-141 86 Huddinge (SE).			
(74) Agent: DEAN, John, Paul; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2JT (GB).			

(54) Title: ORPHAN RECEPTOR

(57) Abstract

This invention relates to a novel estrogen receptor-related nuclear receptor, hereinafter termed "ER β " having the amino acid sequence of Figs. 1, 13A or 14A or substantially the same amino acid sequence as the amino acid sequence shown in Figs. 1, 13A or 13B or an amino acid sequence functionally similar to that sequence. The invention also relates to DNA sequences encoding the receptor. The receptor may be useful in isolating molecules for the treatment of disorders such as prostate cancer, benign prostatic hyperplasia, osteoporosis or cardiovascular disorders and in the testing of substances for estrogenic and other hormonal effects.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Libera	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Larvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Maunitania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/03933

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/12 C12Q1/68 C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 15, no. 6, 25 March 1987, pages 2499-2513, XP002026307 KOIKE, S. ET AL.: "Molecular cloning and characterization of a rat estrogen receptor cDNA" see the whole document ---	1,3-6,8, 9,12
X	SCIENCE, vol. 231, no. 4742, 7 March 1986, pages 1150-1154, XP000611679 GREENE G L ET AL: "SEQUENCE AND EXPRESSION OF HUMAN ESTROGEN RECEPTOR COMPLEMENTARY DNA" see the whole document ---	1,3-6,8, 9,12 -/-

 Further documents are listed in the continuation of box C Patent family members are listed in annex.

* Special categories of cited documents :

- *'A' document defining the general state of the art which is not considered to be of particular relevance
- *'E' earlier document but published on or after the international filing date
- *'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *'O' document referring to an oral disclosure, use, exhibition or other means
- *'P' document published prior to the international filing date but later than the priority date claimed

- *'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- *'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

- *'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

- *'&' document member of the same patent family

2 Date of the actual completion of the international search

Date of mailing of the international search report

27 February 1997

10.03.97

Name and mailing address of the ISA

 European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.
 Fax (+ 31-70) 340-3016

Authorized officer

Kania, T

INTERNATIONAL SEARCH REPORT

National Application No

PCT/EP 96/03933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 17, no. 14, 25 July 1989, pages 5477-5488, XP002026308 LEES J. ET AL.: "Identification of two transactivation domains in the mouse oestrogen receptor" see the whole document ---	1,4-6,8, 9,12
P,X	PNAS U.S.A., vol. 93, no. 12, 11 June 1996, pages 5925-5930, XP002026309 KUIPER G. ET AL.: "Cloning of a novel estrogen receptor expressed in rat prostate and ovary" see the whole document ---	1-10,12
P,X	FEBS LETTERS, vol. 392, no. 1, 19 August 1996, pages 49-53, XP002026310 MOSSELMAN, S. ET AL.: "ERbeta: identification and characterization of a novel human estrogen receptor" see the whole document ---	1-12
T	MOLECULAR ENDOCRINOLOGY, vol. 10, no. 11, November 1996, pages 1293-1307, XP000645166 ENMARK E. AND GUSTAFSSON J.: "Orphan nuclear receptors- the first eight years" see the whole document -----	1-12